EXHIBIT 5

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Karyotypic analysis of methotrexate-resistant and sensitive mouse L5178Y cells

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Abstract. A lymphoblastic leukemia cell line, L5178YR, that is over 100,000-fold resistant to methotrexate (MTX) has been developed. Previous work has demonstrated that the resistant line has dihydrofolate reductase (DHFR) levels 300-fold higher than the sensitive parental cells, L5178Y. Resistant cells grown in the absence of MTX (L5178YR−) showed a similar level of DHFR activity. The increase in DHFR could be entirely accounted for by a corresponding increase in DHFR-specific mRNA and gene copies. Studies were carried out to determine whether these changes in the resistant cells were accompanied by karyotypic alterations. A detailed karyotypic analysis of the sensitive parental line (L5178YS) and the two resistant cell lines (L5178YR+ and L5178YR−) was performed. Although certain abnormal marker chromosomes were present in all three cell lines, the most striking consistent difference between the resistant and sensitive cells was the presence of a large, faintly banded region of intermediate staining intensity, termed a "homogeneously staining region" (HSR), inserted within a reduplicated part of chromosome 2. It was present in approximately 90% of resistant cells, and no more than one HSR was ever present in a cell. Hybridization in situ was performed to determine the chromosomal locations of *DHFR* genes. Utilizing a purified complementary DNA probe made from messenger RNA of the L5178YR+ cells, the genes were shown to be localized exclusively to the HSR.

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Although it is possible to achieve temporary remissions in a number of human cancers, few can be considered curable. Since most cancers eventually become refractory to chemotherapy, the development of drug resistance within tumor populations remains a major concern in the treatment of

cancer (Bertino et al., 1972; Skipper et al., 1978).

A wide variety of biochemical mechanisms have been demonstrated, both experimentally and clinically, whereby tumor cells become resistant to the cytotoxic effects of various drugs (Bertino et al., 1972; Brockman, 1974). Work has also been directed at correlating the development of drug resistance with cytogenetic changes in tumor cells (Hauschka, 1958; Vogt et al., 1959; Harris and Ruddle, 1960; Hakala et al., 1962; Harris, 1964; Hoshino et al., 1966). If biological alterations such as resistance could be correlated with specific chromosomal modifications, general responsiveness of the tumor could be predicted by karyotype analysis.

Biedler et al. (1972) provided the first demonstration of cytogenetic changes related to the development of drug resistance. Studying cell lines derived from normal Chinese hamster tissue that were resistant to methotrexate (MTX) and another antifolate derivative, methasquin (MSQ), the investigators noted the presence of unusually large chromosomes not present in the sensitive parental cell lines. With the use of Giemsa-trypsin banding techniques, they showed that these large chromosomes contained a long segment that did not band, which they termed a homogeneously staining region (HSR). The HSR was found only in MTX- or MSQ-resistant lines with elevated levels of dihydrofolate reductase (DHFR), but its significance remained unclear (Biedler and Spengler, 1976*a*, *b*).

Using molecular hybridization techniques, Alt et al. (1976) studied an MTX-resistant murine sarcoma line (S-180) with elevated levels of DHFR. They determined that the increase in DHFR could be entirely accounted for by an increased rate of its synthesis and demonstrated that there is a corresponding increase in both mRNA and gene copies specific for DHFR (Kellems et al., 1976; Alt et al., 1978). Using a purified mouse ^{3}H-cDNA probe specific for DHFR mRNA from MTX-resistant S-180 cells, the authors demonstrated by hybridization in situ that the HSR of an MTX-resistant Chinese hamster ovary (CHO) cell line contained amplified *DHFR* genes (Nunberg et al., 1978; Schimke et al., 1978). We have studied an MTX-resistant lymphoblastic murine leukemia cell line, L5178YR, with a 300-fold elevation of DHFR. Using similar techniques, this cell line has also been shown to have a corresponding increased rate of DHFR synthesis, mRNA, and genes coding of DHFR. Hybridization in situ studies also demonstrated that amplified *DHFR* genes were located on an HSR (Dolnick et al., 1979). In this communication we report a more detailed karyotypic analysis of the sensitive and resistant MTX sublines of this tumor.

Materials and methods

Materials and methods are described elsewhere in detail (Dolnick et al., 1979). Briefly, L5178Y cells (Hauschka et al., 1958) were grown in increasing concentrations of MTX to select for a population of MTX-resistant tumor cells. The resistant cell line was either continuously maintained in the presence of high concentrations of MTX (i.e., 10^{-3} M), designated R+, or maintained in culture medium without MTX, designated R−, for over 150 cell doublings. R+ and R− cells had identical levels of DHFR activity and of mRNA specific for DHFR at the time when karyotypic analyses were carried out. Both resistant cell lines as well as the sensitive parental line were karyo-

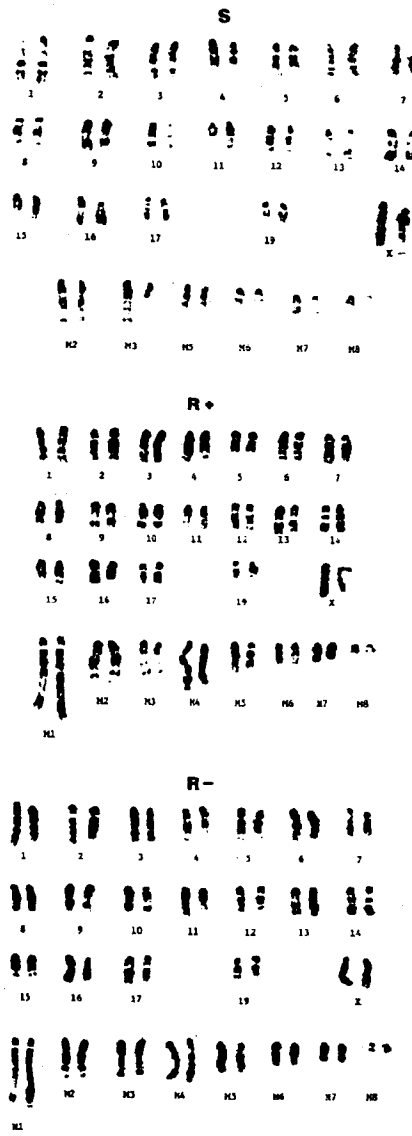

Fig. 1. a. Composite showing two examples of each of the different chromosomes present in L5178YS cells. The chromosomes are derived from more than one spread in order to illustrate the typical banding patterns that permitted the identification of each chromosome. The presumably normal mouse chromosomes (top three rows) are numbered according to the standard nomenclature. One of the two chromosomes 16 had a very large heterochromatin region, while the other No. 16 had a small one. Examples of both types are shown. The characteristics and possible derivation of the rearranged "marker" chromosomes are summarized in table I. The actual number of copies present for each chromosome varied somewhat from cell to cell (table II). An example of a typical metaphase spread has been published (Dolnick et al., 1979). **b.** Composite, derived from more than one metaphase spread, showing two examples of each of the chromosomes present in L5178YR+ cells. The actual frequency of each chromosome varied slightly from cell to cell (table II). An example of a metaphase spread has been published (Dolnick et al., 1979). **c.** Composite of two examples of each of the chromosomes present in L5178YR− cells.

Table I. Characterization and interpretation of marker chromosomes

Marker	Description
M1	Chromosome 2 containing HSR inserted into duplicated region 2E
M2	Chromosome 4 with an interstitial tandem duplication distally
M3	Chromosome 11 with a terminal addition
M4	Chromosome 13 with an extra chromosomal region of unknown origin
M5	Chromosome 7 containing an inversion
M6	Proximal two thirds of a chromosome 14
M7	Possibly derived from a chromosome 18 by inversion
M8	Small chromosome of unknown origin

Table II. Results of detailed karyotype analysis of 12 metaphase spreads

Cell line	Number of copies of each chromosome																		
	Normal chromosomes																		Markers
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19	X
L5178YS																			
1	2	2	2	1	2	2	1	2	2	2	1	1	2	1	1	2	2	3	1
2	2	2	2	1	2	2	1	1	2	2	1	2	2	1	1	2	2	3	1
L5178YR+																			
1	2	1	2	1	2	2	1	2	2	2	1	2	–	1	1	2	2	3	1
2	1	1	2	1	2	2	1	2	2	1	1	2	1	1	1	1	1	3	1
3	2	1	2	1	2	2	1	2	2	2	1	2	–	1	1	2	2	2	1
4	2	1	2	1	2	2	1	2	2	2	–	2	2	1	1	2	2	3	–
5	2	1	2	1	2	2	1	1	2	2	1	2	1	1	1	1	2	3	1
L5178YR−																			
1	2	1	2	1	2	1	1	2	2	2	1	2	–	1	1	1	2	3	1
2	2	1	2	1	2	1	1	2	2	1	1	2	1	2	1	1	2	3	–
3	1	1	2	1	2	2	1	2	1	2	1	2	1	1	1	2	2	3	–
4	2	1	2	1	2	2	1	1	2	2	1	2	1	1	1	2	2	3	–
5	1	1	2	1	2	2	1	2	2	2	1	2	2	1	1	1	2	3	1

Markers								
M1	M2	M3	M4	M5	M6	M7	M8	Unidentified
–	1	1	–	1	1	1	1	1
–	1	1	–	1	–	1	1	3
1	1	–	–	1	1	1	1	4
1	1	1	–	1	1	1	1	4
1	1	1	1	1	1	1	1	3
1		1	1	1	1	1	1	0
1	1	–	1	1	1	1	1	2
1	1	1	1	–	1	1	1	5
1	1	1	1	1	1	1	1	4
1	1	–	1	1	1	1	1	5
1	1	1	–	–	–	1	1	6
1	1	1	–	–	2	1	1	3

typed utilizing trypsin-Giemsa banding (SUN et al., 1974) and standard mouse chromosome nomenclature (NESBITT and FRANCKE, 1973). One hundred metaphase spreads from each cell line were analyzed to establish modal chromosome numbers; the degree of tetraploidy was estimated by analysis of 300 spreads. Hybridization in situ was performed on the R+ cells utilizing a purified ^{3}H-cDNA probe for DHFR mRNA, as described elsewhere (DOLNICK et al., 1979).

Results

Initial chromosome counts suggested that mouse lymphoma cell line L5178Y as well as the MTX-resistant derivatives R+ and R− were diploid. The normal diploid mouse chromosome number of 40 was found in 84/100 L5178YS cells, 66/100 R+ cells, and 73/100 R− cells.

The percentage of tetraploid cells was low in all three cell lines (1%, 2%, and 3% respectively). A large marker chromosome (M1) was present in 90/100 R+ cells and 89/100 R− cells but was not seen in any of the 100 metaphase spreads analysed from the sensitive cell line. Giemsa banding revealed within M1 a large chromosome region of intermediate staining intensity containing a pattern of narrow grey bands. Since this region resembled the "*h*omogeneously *s*taining *r*egions" described by BIEDLER and SPENGLER (1976*a*, *b*) in MTX-resistant Chinese hamster cell lines, it was designated HSR.

A more detailed analysis of the chromosome content in the three types of cells was undertaken to look for other significant karyotypic differences. While most of the chromosomes were apparently structurally intact displaying banding patterns consistent with normal mouse chromosomes, seven rearranged "marker" chromosomes, in addition to the HSR-containing M1, were readily identified. Figures 1*a*, *b*, and *c* are composites rather than actual karyotypes of individual cells, illustrating with two copies of each chromosome, derived from the same or from different cells, the characteristic patterns used for chromosome identification. Figure 1*a* represents the L5178YS MTX-sensitive line, while 1*b* and *c* represent the resistant sublines (R+ and R−, respectively).

Descriptions and possible derivations of the marker chromosomes are summarized in table I. All the markers, with two exceptions, were present in single copies in all three cell lines. M1 and M4 were only found in the resistant lines. Actual karyotype information is summarized for 12 metaphase spreads in table II.

M1 with the HSR was present in all R− and R+ cells and absent in S cells. This correlated with the absence of the second normal chromosome 2 in all R− and R+ cells, while two normal chromosomes 2 were present in S cells. No normal chromosome 18 was seen in any of the three cell lines. M7, however, may represent an inverted 18. M4, derived from a chromosome 13, was present inconsistently, but in R− and R+ cells only. The modal number of normal chromosomes 13 had correspondingly declined from two in S cells to one in R− and R+ cells.

The distal half of M4 contains chromosomal material of unknown derivation that is distinctly banded, although the presence of a small region with "HSR-like" qualities cannot be excluded.

The category of "unidentified" chromosomes (table II) includes heterogenous and inconsistent rearrangements, often unique to individual cells, the frequency of which appeared to increase with time in culture.

No more than one HSR was observed in a single cell. In one spread (of over 100 total) of the R− cells, a translocation of the HSR to chromosome 7 was noted. A rather unusual fusion of at least two copies of the HSR containing chromosome M1, was observed in a tetraploid resistant cell.

In fig. 2, the normal chromosome 2 and M1 are presented diagrammatically using recommended nomenclature for chromosome bands in the mouse (NESBITT and FRANCKE, 1973). This diagram shows that the HSR is located between two sets of

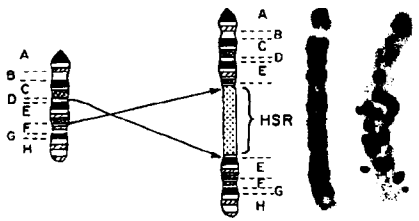

Fig. 2. Ideogram of a normal mouse chromosome 2 (NESBITT and FRANCKE, 1973) and diagram illustrating the most likely derivation of the M1 marker chromosome. The HSR appears to be located within a reduplicated interstitial segment of chromosome 2 (region E). A subtle pattern of repeated grey bands is seen within the HSR. Enlargement of M1 from hybridization in situ experiment demonstrates clustering of silver grains in region corresponding to the HSR.

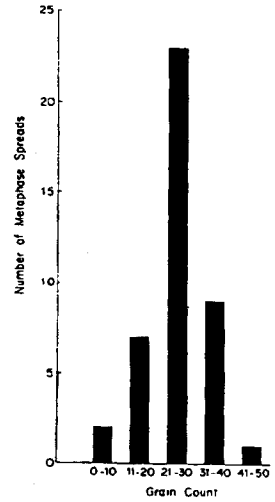

Fig. 3. Distribution of the number of silver grains over the HSR of chromosome M1 in 42 spreads.

reduplicated region 2E. This interpretation represents a refinement of a previously published version (DOLNICK et al., 1979).

Hybridization in situ using ^{3}H-cDNA has localized the amplified *DHFR* genes to the HSR within M1 (fig. 2) (DOLNICK et al., 1979). Figure 3 demonstrates the distribution of silver grains over the HSR in 42 metaphase spreads. The other chromosomes had an average grain count of 1.2 ± 0.05. Further examination of eight typical metaphase spreads showed that the vast majority of chromosomes (besides M1) had either zero or one silver grain, while only three of these eight spreads actually had a chromosome (other than M1) with greater than four grains. Furthermore, in those chromosomes that contained more than one grain, the grains were randomly distributed (data not shown).

These results clearly show that the HSR on chromosome M1 specifically contains the amplified genes for DHFR and no other chromosomal region contains *DHFR* genes detectable by hybridization in situ. However, since the hybridization in situ technique is not sensitive enough to localize unique gene sequences, it is possible that single genes for DHFR exist outside the HSR.

Discussion

HSR's have been found in two different types of cells: (1) antifolate (MTX and MSQ)-resistant cell lines with elevated DHFR (BIEDLER and SPENGLER, 1976; NUNBERG et al., 1978; BOSTOCK et al., 1979) and (2) certain tumor cell lines with no evidence of drug resistance, e.g., human neuroblastoma (BIEDLER and SPENGLER, 1976*a*, *b*; BALABAN-MALENBAUM and GILBERT, 1977),

other human malignancies (KOVACS, 1979), and in a murine adrenal carcinoma line (GEORGE and FRANCKE, 1980). The studies reported here and elsewhere (NUNBERG et al 1978; DOLNICK et al., 1979) demonstrate the relationship of these regions to gene amplification in the MTX-resistant cell lines, but their possible significance in tumor cell lines is unclear and must await further investigation.

Comparison of HSR's in various cell lines demonstrated certain qualities common to all HSR's, while other features appear to vary between different cell lines. The location of the HSR on the chromosome has been found to be interstitial in neuroblastoma cell lines (BIEDLER and SPENGLER, 1976; BALABAN-MALENBAUM and GILBERT, 1977), MTX-resistant Chinese hamster ovary cells (NUNBERG et al., 1978), and L5178Y cells (DOLNICK, 1979). In contrast, the antifolate resistant sublines of BIEDLER and SPENGLER (1976*a*, *b*) and BIEDLER et al. (1974) have contained HSR's in a terminal location on the chromosome. In some neuroblastoma and MTX-resistant cell lines, the HSR has been found to be present exclusively in one chromosomal location (NUNBERG et al., 1978; DOLNICK et al., 1979), while in other cell lines the HSR has been found on different chromosomes. However, the HSR's appeared to be localized to a specific region of each HSR-containing chromosome, which was invariant within any given cell line. The 300-fold elevation of DHFR in the L5178Y MTX-resistant cells is consistent with an earlier observation by BIEDLER et al. (1974) that only cells with a greater than 100-fold increase in DHFR showed an HSR in MTX- and MSQ-resistant Chinese hamster cell lines. This is the first study in which localization of an amplified gene to an HSR has been accomplished in a completely homologous system. The ^{3}H-cDNA probe was prepared from the same resistant cell line in which the HSR was discovered. In situ hybridization studies demonstrated that the multiple *DHFR* genes were located within the HSR (DOLNICK et al., 1979). Furthermore, the lack of clustering of silver grains to other chromosomes excluded the possibility that a significant number of *DHFR* genes were found on other chromosomes (elsewhere in the genome). Finally, the distribution of silver grains along the complete length of the HSR provided evidence that this entire region contains *DHFR* genes (fig. 2). In the absence of selection pressure, i.e., growth of cells without MTX, the HSR remained stable in size and the frequency of M1 remained unchanged.

Does gene amplification with the concomitant development of an HSR occur only at the chromosomal site of the structural gene? If this were the case, the location of the HSR in MTX-resistant cell lines might provide a clue to the map position of the *DHFR* gene. The present study suggests chromosome 2 as a possible site of *DHFR* in the mouse. However, in a MTX-resistant derivative of mouse L1210 leukemia cells, an HSR was observed to develop at the terminal end of a mouse chromosome 4 after the distal portion of this No. 4 had been translocated to a chromosome 14 in the sensitive L1210 progenitor line (TROWSDALE et al., 1980). Therefore, one of two mouse chromosomes, No. 2 or No. 4, might carry the *DHFR* gene. Unless these lines contain additional undetected rearrangements involving the HSR-bearing chromosomes, it appears that HSR's may be found at sites distant from the structural gene.

It has been our impression, as well as that of other authors (Bostock et al., 1979; Miller et al., 1979; George and Francke, 1980), that HSR's are not really "homogeneously staining" or "unbanded." Instead, with trypsin-Giemsa banding techniques a pattern of fine gray bands is usually visible which often gives the impression of multiple repeats of a basic unit. Studying a rat hepatoma cell line, Miller et al. (1979) have termed such regions containing amplified rDNA sequences "differentially stained regions (DSR)." We prefer to use the term HSR because it has become well established in describing this particular cytological phenomenon. Although most HSRs are reported to be lightly staining with C-banding, intermediately with G-banding, and darkly with R-banding, occasional reports of HSR's staining positively with C-banding (Bostock et al., 1979) suggest that these chromosome regions may be heterogeneous, not only with respect to amplified unique DNA sequences, but also with respect to the unspecific repetitive sequences contained in them and their associated chromosomal proteins.

Restriction mapping and DNA sequencing have allowed a more precise picture of the arrangement of multiple genes within these chromosomal regions. Such studies have shown that the structural genes are separated by nontranscribed spacer units presented in tandem array along a portion of the genome (and that the whole basic repeat consists of a structural gene plus spacer unit) varying in length from several hundred to a few thousand base pairs (Tartof, 1975). Although precise mapping data are not yet available for MTX-resistant tumor lines with amplified *DHFR* genes, an estimate of the size of this unit can be made since the HSR corresponds to approximately 5% of the total chromatin. There are estimated to be 4.6×10^9 base pairs of DNA per mouse cell (Lewin, 1974), and therefore, the HSR should have 2.3×10^8 base pairs. Utilizing an average amplification estimate of 300-fold (Dolnick et al., 1979), one can calculate that there are an estimated 800 kilobase pairs in the basic repeat unit. With the actual structural gene for DHFR estimated to be on the order of 40 kilobase pairs (Nunberg et al., 1980), it is obvious that this represents a substantial amount of nonstructural genetic material. It is possible that most of this DNA has no functional significance and simply accumulates during the process of gene amplification. On the other hand, the large amount of DNA in the HSR may play a vital role in the actual process of gene amplification.

The mechanism of gene amplification in MTX-resistant cell lines is still unknown. Possibilities that have been suggested include the involvement of double minutes (small noncentromeric chromosomal fragments) (Kaufman et al., 1979), virally associated reverse transcriptase (Baltimore, 1970), unequal sister chromatid exchanges (Wolff, 1977), and translocatable elements (Kleckner, 1977). Further research will be necessary to elucidate the process of gene amplification.

The implications of the discovery of the HSR are far reaching. To have a marker chromosome for resistance to chemotherapeutic agents has been a major goal of cancer cytogenetics for over 20 years (Biedler, 1975). With refinement of banding techniques, it may not be long before a catalogue of chromosomal alterations exists for many different types of chemotherapeutic resistance. With such chromosomal markers, one could then follow the development of drug resistance and alter therapy accordingly with changes in chromosomal patterns.

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Karyotype analysis and quantitation of viral transforming genes in Rous sarcoma virus transformed, revertant, and retransformed field vole cells

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Abstract. Comparative studies of the number of cellular chromosomes and viral genes, including the gene required for malignant transformation, were performed on several clones of Rous sarcoma virus-transformed, revertant, and spontaneously retransformed field vole cells. The results of these studies indicate that no appreciable differences in either total viral gene equivalents or transforming gene sequences can be detected between transformed and revertant cell types, even though considerable differences in the number of certain chromosomes exist among the clones tested. Furthermore, no increase in the amount of total genes or transforming gene sequences accompanies retransformation of revertant clones, including clones that exhibited significant increases in chromosome number following retransformation.

We have been investigating the status and genetic expression of the Rous sarcoma virus (RSV) genome in infected, transformed field vole *(Microtus agrestis)* fibroblasts in an attempt to obtain information concerning the mechanisms by which avian RNA tumor viruses alter the growth properties of mammalian cells. The field vole cell lines are a particularly useful system to study differences between normal and transformed cells because several RSV-transformed clones have reverted to the normal phenotype without the apparent loss of any detectable portion of the viral genome, including the transforming (sarcoma) gene sequences (KRZYZEK et al., 1978). These revertant vole cells are similar to normal vole cells in their morphology and growth properties. Infectious, transforming RSV can be rescued from the revertant cell lines,

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